Presence of platelet-activating factor-acetylhydrolase in milk

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Abstract Human milk contains numerous factors such as immunoglobulins, lactoferrin, lysozyme, macrophages, etc., which serve an immunoprotective role. Platelet-activating factor (PAF) is one of the most proinflammatory agents thus far described. PAF is metabolized to the biologically inactive lysoPAF by the enzyme PAF-acetylhydrolase (PAF-AH). In the present study we have demonstrated that PAF-AH activity is present in human milk. The activity was associated with aqueous phase and was not stimulated by the addition of bile salts or Ca^{2+}. The activity of PAF-AH in human milk was not affected by the addition of propranolol or NaCl. PAF, and 1-acyl-2-acetyl-glycerophosphocholine were the only substrates cleaved by the enzyme. Based on these properties it is concluded that the milk PAF-AH is not the lipoprotein or bile salt-stimulated lipase known to be present in milk. Inhibitor studies revealed that the enzyme in human milk was the plasma type PAF-AH. The activity of PAF-AH was stable at pH 4.0 at 37°C and the activity varied in milk samples obtained from various species. The enzyme was secreted by milk macrophages. The presence of PAF-AH in human milk may explain, in part, the beneficial effects of breast feeding in the prevention of necrotizing enterocolitis by inactivating the potent proinflammatory autacoid, PAF.—**Furukawa, M., H. Narahara, K. Yasuda, and J. M. Johnston.** Presence of platelet-activating factor-acetylhydrolase in milk. *J. Lipid Res.* 1993. **34:** 1603–1609.

Supplementary key words PAF • milk • macrophages • necrotizing enterocolitis

A number of enzymes have been reported to be present in human milk (1). Two lipase activities have been demonstrated, lipoprotein lipase (LPL) (EC 3.1.1.3) and a bile salt-stimulated lipase (BSSL) (1). The former enzyme is associated with the lipid globules of human milk and the latter is present in the aqueous phase. The function of LPL is thought to be the uptake of the fatty acids from the circulating plasma lipoproteins into the mammary gland. The activity present in human milk may be a reflection of the "leakage" of the enzyme from this tissue (2). The second lipase in human milk, which is stimulated by bile salts (3), is thought to have a more direct function. It has been suggested that the combination of lingual gastric lipase with that of BSSL will facilitate the complete hydrolysis of dietary triacylglcyerols. The BSSL is stable at low pH and thus is not inactivated at the pH present in the gastric juice of the neonate (3).

Platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), has been recognized for some time as a potent proinfiammatory agent (for review see 4). The lipase that inactivates PAF, PAF-AH, shows a specificity for the cleavage of short chain fatty acid esters (5), such as an acetyl group and, more recently, intermediate chain fatty acids that have an oxygen function due to their oxidation at the *sn*-2 position (4). Two distinct forms of the enzyme have been identified, a plasma and an intracellular isozyme (5). The plasma enzyme is associated with the lipoprotein fraction (6) and the activity is increased during a "stress" reaction in the lizard (7), in patients with ischemic cerebrovascular disease (8), in insulin-dependent diabetes (9), and in spontaneous hypertensive rats (10) and in white males with hypertension (5). The activity is decreased in a family of asthmatics (11). The maternal plasma activity is decreased during pregnancy, but returns to normal levels immediately following delivery. Fetal and newborn animals and infants have a lower activity of plasma PAF-AH than adults (12). The properties of PAF-AH in human disease have been recently reviewed (13). In view of these findings and in consideration of the fact that PAF-AH activity is present in association with various lipid fractions, we have examined the activity of this enzyme in milk.

Abbreviations: PAF, platelet-activating factor; AH, acetylhydrolase; LPL, lipoprotein lipase; GPC, glycero-3-phosphocholine; BSSL, bile salt-stimulated lipase; *p*-BPB, *p*-bromophenacylbromide; NEC, necrotizing enterocolitis.

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MATERIALS AND METHODS

Materials

[^{3}H]PAF (1-hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine) (sp act 10 mCi/μmol) was purchased from DuPont-New England Nuclear (Boston, MA). Unlabeled PAF was obtained from Avanti Polar Lipids (Pelham, AL) and purified by thin-layer chromatography on silica (Merck), using chloroform–methanol–acetic acid–water 50:30:8:6 as the mobile phase. 1-Palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, 1-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1-oleoyl-2-acetyl-*sn*-glycero-3-phosphocholine, trypsin, dithiobisnitrobenzoic acid (DTNB), and *p*-bromophenacylbromide (pBPB), were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium fluoride (NaF) was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ).

Milk samples

The human breast milk samples were obtained by direct expression or via a breast pump. The rat milk samples were obtained after pitocin administration to lactating rats. The milk was expressed by the application of intermittent vacuum. The cow and goat samples were obtained from a local dairy prior to pasteurization. Some samples were frozen immediately and stored at −20°C prior to use. In preliminary experiments, it was demonstrated that samples could be stored as long as 3–4 months under these conditions without loss of activity. PAF-AH was assayed directly or after centrifugation at 600 *g* for 10 min at 4°C. After centrifugation, a fatty layer (upper), aqueous layer (middle), and small pellet fraction were obtained. In some instances the fractions were stored at −20°C until assayed. We have shown that the enzyme in milk is stable for up to 4 months under these conditions.

Macrophage isolation and culture conditions

Human milk macrophages were isolated by adherance by the procedure previously described (14) with minor modifications. Milk was centrifuged at 150 *g* at room temperature for 10 min and the pellet containing the cells was washed two times with Dulbecco's phosphate-buffered saline (pH 7.4, minus Ca^{2+} and Mg^{2+}; D-PBS). Cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) containing 10% (vol/vol) heat-inactivated bovine calf serum (BCS), penicillin (100 units/ml), streptomycin (100 μg/ml), HEPES (25 mM), sodium bicarbonate (30 mM), and L-glutamine (4 mM) (10% BCS-IMDM). The cell suspensions (5×10^4 cells/ml) were allowed to adhere to 96-well culture plates for 30 min at 37°C in humidified 5% CO_2–95% air. Nonadherent cells were removed from the cultured plates by two washes with IMDM. Adherent cells were cultured in 0.2 ml of 10% BCS IMDM at 37°C in humidified 5% CO_2–95% air. In some experiments, whole cell populations isolated from human milk by centrifugation were cultured under the same conditions as described above. Cells were greater than 95% viable under all experimental conditions as judged by trypan blue exclusion and release of lactate dehydrogenase into the medium. After incubation, the supernatant was collected and stored at −80°C.

Plasma samples

Human plasma was obtained from normal healthy subjects using EDTA as anticoagulant, and stored at −80°C until use. The plasma was diluted 10-fold with 0.25 M sucrose prior to assay.

Cytosolic fraction

Wister rats were killed and the livers were removed and homogenized in 6 vol of 0.25 M sucrose at 4°C using a PT-10/20 Polytron homogenizer (Heat System-Ultrasonics, Inc., Farmingdale NY). The homogenate was centrifuged at 600 *g* for 10 min. The resulting supernatant fraction was further centrifuged at 18,000 *g* for 15 min and then at 105,000 *g* for 60 min. The cytosolic fraction thus obtained was used as the source of the intracellular PAF-AH activity. All procedures were performed at 4°C.

Assay for PAF-acetylhydrolase activity

The activity of PAF-AH in human milk was assayed according to the method of Miwa et al. (11) with minor modifications. The assay mixture contained 300 μl of Tris-HCl (50 mM, pH 7.4) containing BSA (2.0 mg/ml), substrate (0.05 mM, 4.0 Ci/mol, mixture of nonradiolabeled PAF and [^{3}H]PAF), and 100 μl of human milk. The final volume was 500 μl. Human sera of known activity were assayed with each group of samples. Little variation (less than 5%) occurred in these control samples. The assay mixtures were incubated for 20 min at 37°C and terminated by addition of trichloroacetic acid (final concentration 7%). In some experiments, BSA (final concentration 5%) was added at the end of the incubation followed by the addition of TCA.

Characterization of the PAF-acetylhydrolase in human milk

In order to determine whether PAF-AH activity differs from other lipases previously reported in human milk, various bile salts, EGTA, $CaCl_2$, NaCl, and propranolol were also added to the incubation mixture. The sodium salts of taurocholate, glycocholate, or cholate at concentrations between 1 and 12 mM were used to investigate the effect of bile salts on the activity of the enzyme. In order to determine whether or not Ca^{2+}-dependent phospholipase was responsible for the activity present in milk, milk was also preincubated with EGTA (10 mM) or $CaCl_2$ (10 mM) for 30 min followed by the incubation with [^{3}H]PAF. Similarly, incubations were performed with NaCl (200 mM) and propranolol (2 mM) in order to

differentiate the activities of LPL and PAF-AH. Both compounds have been reported to inhibit LPL (15, 16). In studies, PAF-AH activity was characterized after the preincubation with either DTNB, *p*-BPB, NaF, or trypsin in order to establish whether or not the PAF-AH in milk was the plasma or cytosolic isozyme. PAF-AH activities were assayed as described. Preincubation mixture contained 300 μl Tris-HCl (50 mM, pH 7.4) containing BSA (20 mg/ml), various amounts of human milk, and DTNB (0.75 mM), *p*-BPB (0.5 mM), NaF (20 mM), or trypsin (0.02%). *p*-BPB was dissolved in ethanol and added to the preincubation mixture. The final concentration of ethanol was 0.8%.

pH Stability

Milk samples were preincubated for 30 min at pH between 2.9 and 9.7. After preincubation the pH of mixture was confirmed and the pH was adjusted to 7.4 prior to PAF-AH assay.

RESULTS

Distribution of PAF-AH in milk

The distribution of PAF-AH after centrifugation of human milk at 600 *g* for 10 min was determined. Seven human milk samples obtained from different women were included in this study. Ninety-seven percent of the PAF-AH in the original milk samples was associated with the intermediate or aqueous phases. No significant activity was present in the lipid layer (upper) or pellet fractions. Similar findings were obtained when the milk of other species that contained PAF-AH were processed. LPL is present in lipid-containing upper layers (2). Based on this finding it would appear that the lipase activity in milk is not LPL.

Effect of bile salts on the PAF-AH in milk

In order to determine whether PAF-AH activity differs from BSSL present in human milk, the effect of various bile salts on the activity was examined. When the effects of taurocholate, glycocholate, and cholate were examined at concentrations ranging from 1 to 12 mM, no stimulation of PAF-AH activity was observed at any concentration of bile salt. At lower concentrations of the bile salt, no effect was observed and a 25% inhibition was found at the higher concentration of the bile salts. Previously, it had been reported that only glycocholate was effective in the stimulation of the BSSL (17). If bile salt will alter the hydrolysis of PAF in a manner similar to the long chain substrates, it is concluded that the lipase activity observed in milk using PAF as substrate was not that of the previously described BSSL.

TABLE 1. Effect of EGTA, Ca^{2+}, NaCl, and propranolol on milk PAF-AH activity

Additions	Enzyme Activity Remaining
	%
EGTA	97.1 ± 2.8
$CaCl_2$	94.0 ± 3.9
NaCl	95.9 ± 3.8
Propranolol	91.3 ± 4.7

Milk was preincubated with EGTA (10 mM), $CaCl_2$ (10 mM), NaCl (200 mM), and propranolol (2 mM) for 30 min at 37°C prior to assay of enzyme activity. The control activity was 1.0 nmol/min per ml in the absence of any addition. Three separate samples were analyzed.

TABLE 2. Substrate specificity of PAF-AH activity in human milk

Additions	Acetylhydrolase Activity	
	Milk	Plasma
	%	
None	100.0	100.0
PAF	57.6 ± 6.2	52.4 ± 2.8
1-Palmitoyl-2-arachidonoyl-GPC	97.3 ± 4.0	94.0 ± 6.7
1-Hexadecyl-2-oleoyl-GPC	94.9 ± 5.3	93.4 ± 5.5
1-Oleoyl-2-acetyl-GPC	84.6 ± 9.9	62.3 ± 2.8

Nonradiolabeled PAF or various glycerophospholipid analogs (25 nmol) were added as an albumin complex to the assay mixture containing 0.01 nmol of [^{3}H]PAF. Results are expressed as percentage of the remaining activity of a nontreated control. Values shown are the means ± SD of five experiments in which duplicate assays were carried out for each substrate. GPC, glycero-3-phosphocholine.

Effect of various EGTA, Ca^{2+}, NaCl, and propranolol on PAF-AH in milk

In order to determine whether PAF-AH in milk had properties similar to phospholipase A_2 or LPL, the effects of various known inhibitors and activators of these two enzymes on PAF-AH activity in human milk were examined. $CaCl_2$ (10 mM) and EGTA (10 mM) were incubated with human milk for 30 min prior to assay. As can be seen in **Table 1,** the PAF-AH activity was not affected by the addition of Ca^{2+} or EGTA. The failure to observe a stimulation by Ca^{2+} or an inhibition by EGTA is suggestive that the PAF-AH in milk does not have the properties of a Ca^{2+}-dependent phospholipase A_2. It has been reported by Kubo and Hostetler (16) that propranolol (2 mM) and NaCl (200 mM) will inhibit LPL. As can be seen from the table, neither propranolol nor NaCl were inhibitory for the activity when preincubated with the human milk enzyme. Based on these findings, as well as the fact that PAF-AH activity was present in the aqueous phase where LPL is found in the lipid upper phase, it is concluded that the PAF-AH activity in milk is not due to a Ca^{2+}-dependent phospholipase A_2 or LPL.

Substrate specificity

Substrate competition studies were used to establish the specificity of the PAF-AH present in milk. The substrate specificity was ascertained by the addition of various non-radioactive analogs to the reaction mixture at concentrations equal to that of [^{3}H]acetyl-PAF. The compounds added were 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, 1-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine, and 1-oleoyl-2-acetyl-*sn*-glycero-3-phosphocholine. The various substrates were complexed to albumin. The substrates containing long chain fatty acid moieties (i.e., oleoyl or arachidonoyl) in the *sn*-2 position and an alkyl or acyl groups at the *sn*-1 position were incubated in the presence of an equal molar concentration of [^{3}H]acetyl PAF (**Table 2**). None of the substrates containing a long chain fatty acid ester in the *sn*-2 position altered the hydrolytic rate of PAF. When 1-acyl-2-acetyl-GPC was added, however, this substrate did reduce the hydrolytic rate of radiolabeled PAF. A similar substrate specificity was observed when porcine milk was used (data not shown). These observations are consistent with the known properties of PAF-AH (13, 18).

PAF-AH type in milk

Two distinct PAF-AH types have been characterized, the cytosolic and plasma type (4, 5). In order to establish whether the PAF-AH activity in milk was the plasma or intracellular isozyme, the PAF-AH activities obtained from both human and porcine milk were examined in the presence of various known effectors of the two isozymes. For comparison, the activity in milk was compared to the rat liver cytosolic fraction and human plasma. The effects of *p*-BPB, DTNB, NaF, and trypsin on the activities are summarized in **Table 3.** The PAF-AH in human serum has been reported to be insensitive to all of these reagents while the cytosolic enzyme is sensitive. As is seen in Table 3, the enzyme in milk had properties almost identical to those of the human plasma enzyme.

TABLE 3. PAF-AH type in milk

Additions	Enzyme Activity Remaining		
	Milk	Plasma	Cytosol
	%		
Trypsin	87.1 ± 6.9	84.8 ± 6.4	20.5 ± 10.5
DTNB	89.1 ± 2.4	93.7 ± 2.0	33.5 ± 9.6
NaF	89.2 ± 2.4	92.3 ± 2.1	41.5 ± 5.0
p-BPB	94.6 ± 3.5	98.0 ± 4.5	43.8 ± 0.6

Samples were preincubated with either trypsin (0.04%), DTNB (1.5 mM), NaF (40 mM), or *p*-BPB (0.15 mM) for 30 min at 37°C and the PAF-AH activity was assayed as described. A 5% rat liver cytosolic preparation was used for the comparison of the intracellular and plasma PAF-AH activities. Results are expressed as percentage of the remaining activity of a nontreated control. Values shown are the means ± SD of three experiments in which assays were performed in duplicate.

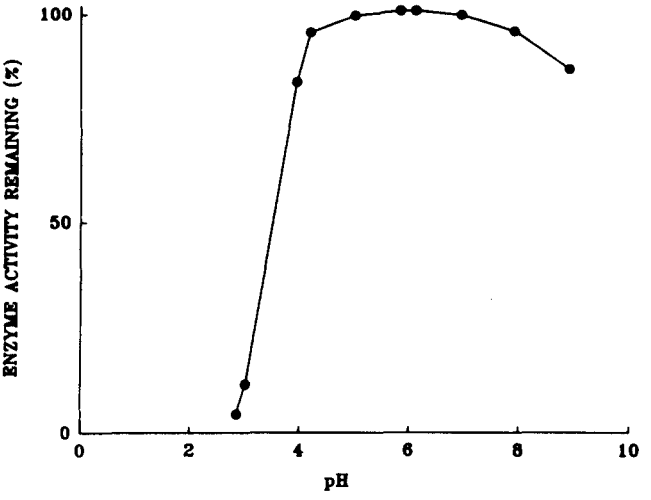

Fig. 1. pH Stability of human milk PAF-AH. Milk (250 μl) was preincubated with 250 μl of the following buffers prior to PAF-AH assay: citrate-phosphate (0.1–0.2 M; pH 2.2–5.0), phosphate (0.2 M; pH 6.0–8.0), glycine-NaOH (0.2 M; pH 9.0–10.0). A representative experiment is illustrated. The experiment was repeated three times with minimal change.

pH Stability of PAF-AH in milk

The pH stability of the enzyme was also examined. The results are illustrated in **Fig. 1.** At pH values below 3.0 the enzyme was inactivated. At higher pH values, up to 9, however, the enzymatic activity was not altered. The stability at pH values between 4 and 9 may be of importance in maintaining the activity of the enzyme in the small intestine after transport through the stomach of the neonate.

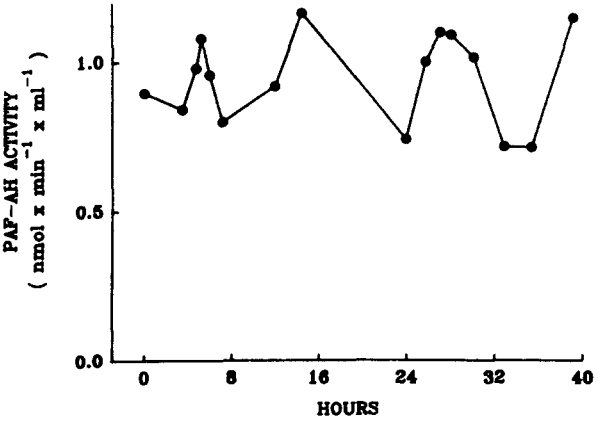

Fig. 2. Diurnal variation of PAF-AH activity in human milk. Milk samples were collected from one mother before each feeding on 2 consecutive days. Samples were stored at −20°C until assayed. Sample one (0 h) was collected at 8 AM

Diurnal and in-feed levels of PAF-AH activity in human milk

Milk samples were collected from one mother before each feeding on 2 consecutive days. It has been reported that there is a diurnal variation in the activity of LPL in human milk, being elevated at noon (19). The BSSL activity, on the other hand, shows very little variation with respect to the time of obtaining the samples (19). As is seen from **Fig. 2,** the PAF-AH activity peaks at 12 AM and 12 PM. We have also examined the enzyme activity in the 10 ml of milk obtained at the beginning and the end of an individual feeding period. No change in the specific activity for PAF-AH was found (data not shown).

PAF-AH activity in milk from various species

We examined PAF-AH activity in milk from various species (**Table 4**). The specific activity of PAF-AH was similar in milk samples obtained from the human, pig, goat, and the rat. The activity was low in the sheep and extremely low to undetectable in the cow. Cow's milk obtained at various times post-partum was also analyzed as well as colostrum. The PAF-AH was absent or present in only trace amounts in all samples obtained from the cow. Colostrum samples were also obtained from the sow. The colostrum activity in this species was 10 nmol × min^{-1} × ml^{-1} and the milk sample at 1 week was 4 nmol × min^{-1} × ml^{-1}. It has been concluded that PAF-AH is present in a number of species including the human but absent in cow's milk.

Cellular origin of milk PAF-AH

Elstad et al. (20) have previously reported that PAF-AH was secreted by human peripheral macrophages. The secreted enzyme was shown to be the plasma type isozyme. The enzyme is also secreted by alveolar macrophages (21) as well as by HL-60 cells (22) treated with phorbol esters. The PAF-AH secreted by these cells as well as by HepG2 cells (23) is the plasma type enzyme. Macrophages are known to be present in human milk (14). The macrophages from human milk were therefore isolated by the procedure described and the PAF-AH secreted into media was determined. The results of these studies are illustrated in **Fig. 3.** As can be seen, a significant release of PAF-AH into the media was observed when the macrophages isolated from human milk were used. The secreted enzyme was shown to be the plasma type by the use of various effectors previously used (data not shown). It was, therefore, concluded that the PAF-AH in milk was, at least in part, derived from the milk macrophages.

TABLE 4. PAF-AH activity in milk from various species

Species	PAF-AH Activity
	nmol × min^{-1} × ml^{-1}
Human	3.4 ± 2.8 (7)
Pig	4.7 ± 2.6 (6)
Goat	4.0 ± 1.9 (3)
Rat	5.8 ± 2.6 (4)
Sheep	0.5 ± 0.02 (3)
Cow	< 0.2 (40)

Milk samples were subjected to centrifugation as described in Materials and Methods and stored at −20°C until assayed. Number of samples analyzed shown in parentheses.

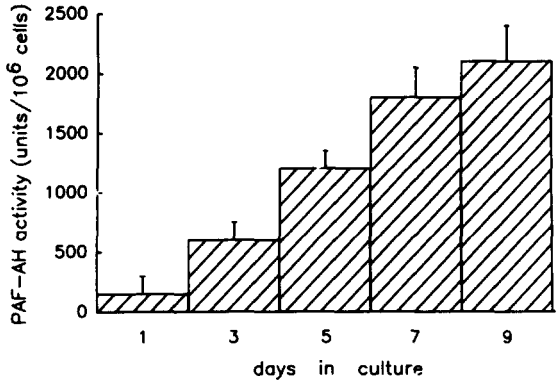

Fig. 3. Time course of PAF-AH secretion by human milk macrophages. Macrophages were isolated from human milk and cultured for 9 days. The PAF-AH activity secreted into the medium was assayed.

DISCUSSION

The presence of LPL in the milk of various species has been known for a number of years (2). The physiological role of this lipase has not been established and it has been suggested that LPL activity may be due solely to its release from the endothelial cells of mammary tissue in association with milk production. The BSSL, on the other hand, appears to have an important function in the digestion of the milk triacylglycerols (1). This enzyme has been shown to be stable after exposure to acidic pH for limited periods of time (3) and serves to supplement the pancreatic lipase activity. The role of the lipase activities described in milk has been primarily concerned with the lipolysis of triacylglycerols. It was recently suggested, however, that the BSSL had a "killing effect" on a widely distributed protozoan parasite that is frequently associated with a diarrheal disease (24, 25).

PAF is one of the most proinflammatory agents thus far described and has been implicated in the development of certain inflammatory diseases of the small and large intestine (26). The major mechanism by which PAF is inactivated is by the removal of the acetate residue of PAF with the formation of lysoPAF. It has been suggested that pasteurization of fresh milk, in addition to its positive role, may also have certain negative consequences (27). A

possible additional negative effect might be the inactivation of the enzyme PAF-AH.

Recently, Caplan and colleagues (28) have suggested a role for PAF along with endotoxin and TNFα in the development of necrotizing enterocolitis (NEC) of the newborn. These investigators have reported that the PAF concentration in plasma was elevated and the PAF-AH significantly lower in patients with this disease. Gonzalez-Crussi and Hsueh (29) also developed a model for this disease in which PAF was one of the administered components that facilitated the development of NEC. PAF administration has also been shown to induce colitis in a model system developed by Wallace and Whittle (30). In *E. coli*, the synthesis of PAF from lysoPAF has recently been reported (31). This observation may account for the presence of PAF in the intestinal lumen.

The beneficial effects of breast feeding compared to formula feeding in the prevention of NEC in the human has been reported by several groups (32, 33). The presence of PAF-AH in raw human milk may contribute to its greater protection. In a canine model system for NEC, it was reported that raw cow's milk was not a satisfactory substitute for the nursing pup (34). The absence of PAF-AH in bovine milk may contribute to this difference. The observation that the PAF-AH of human milk is resistant to exposure to a low pH may also be important in the prevention of PAF accumulation in the small intestine as PAF-AH would escape inactivation in the stomach of the neonate. It has also been reported that the presence of the white cells of milk may be beneficial in the prevention of this disease (35). The presence of PAF-AH in human milk and the demonstration that this enzyme could be secreted by the macrophage population of human milk may explain, in part, the beneficial effects of nursing on the prevention of the development of NEC.

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